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(57) Abstract

A target sequence including a chromosomal translocation or other abnormality is detected by reacting the target sequence, under hybridising conditions in a homogeneous phase, with excess amounts of capture and reporter oligonucleotide probes which are respectively complementary to different regions of the target sequence; and separating and detecting any resultant hybrid that has both labels.

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ASSAY AND KIT FOR THE DETECTION OF CHROMOSOMAL ABNORMALITIES

Field of the Invention

This invention relates to a process and kit for use in detecting the nucleic acid sequences that occur in chromosomal abnormalities.

Background of the Invention

Chromosomal abnormalities are the cause of various undesirable conditions in humans, both inherited and non-inherited, including neoplastic conditions such as follicular lymphoma. It is clearly of vital importance to detect such abnormalities, whether caused by chromosomal translocation, transposition, intergene or intragene recombination, insertion, deletion or point mutation at an early stage by a simple and reliable test.

The association between translocations and pathological states, e.g. neoplastic degeneration, is described by Russo et al, in "Recent Advances in Hematology", A.V. Hoffbrand (ed.), 5, 121-130, Churchill Livingstone. More particularly, a translocation t(14;18) that involves portions of the genes bcl-2 and J_H has been strongly correlated to human follicular lymphoma; see Tsujimoto et al (1985) Science 228: 1440-1443, and Science 229: 1390-1393; Stetler-Stevenson et al (1988) Blood 72: 1822-1825; and Crescenzi et al (1988) Proc. Natl. Acad. Sci. U.S.A. 85: 4869-4873.

Follicular lymphoma (FL) is a B-cell disorder which is related to the presence of a genetic abnormality called the bcl-2 translocation. About 90% of follicular B-cell lymphomas and 20% of large diffuse B-cell lymphomas carry the t(14;18)(q32;q21) translocation which directly involves the IgH locus on chromosome 14 and the bcl-2 locus on chromosome 18. Analogous to the myc translocations in Burkitt's lymphoma, the t(14;18)(q32;q21) translocation occurs 5' or 3' to the bcl-2 gene, but not within the protein coding portion of the gene. It appears that in FL the translocation takes place in pre-B-cells during the

recombination of the J_H region in the IgH chain locus. The association of the bcl-2 oncogene with the heavy chain locus results in high levels of bcl-2 expression.

The FL translocations are structurally uniform. In about 70% of human FL the breakpoints are clustered within the 3' untranslated region of the gene, designated "Major Breakpoint Region" (MBR). In another 10-20% of the cases, the breakpoints are clustered in a region more than 20 Kb downstream from bcl-2's second exon, designated "minor cluster region" (mcr). In some cases translocations have been detected near the 5' exon.

Currently available methods for the detection of the bcl-2 translocation rely on cytogenetic assays (karyotype analysis, which however cannot distinguish between MBR and mcr) or on DNA digestion with restriction enzymes and subsequent Southern blotting, usually involving the use of radioactive probes.

In recent years, many methods for identifying nucleic acid sequences have been developed. They are generally solid-phase methods and relatively rapid and easy to carry out, but difficult to quantitate and not easily adaptable for clinical and diagnostic laboratories. If radioactive labelling is avoided, for ease of operation, it is at the expense of the sensitivity of the method. This drawback can be overcome by amplifying the sequence to be detected, e.g. using the polymerase chain reaction (PCR) as disclosed in EP-A-0200362 and EP-A-0258017.

GB-A-2169403 describes a method for the identification of nucleic acids, in which two independently-labelled oligonucleotide probes are reacted in a single solution, under hybridising conditions, with a target analyte. If the analyte contains a sequence that hybridises to both probes, this may readily be detected by virtue of the fact that one label allows separation of the hybrid and the other its detection. The same or similar techniques are described in, for example, EP-A-0128332, EP-A-0145356, EP-A-0159719, EP-A-0177191, EP-A-0192168 and EP-A-0198662.

Oligonucleotide probes and their use in detecting chromosomal abnormalities are described in, for example, US-A-4701409, US-A-5015568, US-A-5024934, EP-A-0181635 and EP-A-0252685.

Summary of the Invention

According to the present invention, a process of the general type described in GB-A-2169403 is applied to the detection of chromosomal abnormalities, e.g. translocations, using capture and reporter probes that are respectively complementary to different regions of the target sequence, e.g. on opposite sides of the translocation. The respective probes, and any other components used in the procedure, as required, may be formulated into a novel kit comprising a plurality of containers in which the components are distributed.

Given the importance of assaying for chromosomal abnormalities, the present invention provides a number of valuable characteristics. Firstly, for example, it is simple to use, e.g. by relatively unskilled personnel in hospitals and less specialised laboratories; it is quick, non-radioactive and requires only simple equipment.

Secondly, the absorbance readings allow a quantitative measurement of the final signal. With other methods, such as gel electrophoresis/Southern blotting, or dot-blotting, this quantitative determination of signal is only possible with the use of sophisticated instrumentation. These traditional methods are much more prone to subjective interpretation. The quantitation of the signal allows much easier comparison of results between experiments, and between laboratories.

Thirdly, the system will only generate a signal if both reporter and capture probes (complementary to sequences on either side of the breakpoint) bind. This provides a very high degree of specificity and helps minimise the risk of false positives; this is particularly important as this technology has primarily been designed to

detect chromosomal translocations associated with malignancies.

The use of two probes, internally "nested" with respect to primers used for amplification by PCR, also reduces the risk of obtaining false positives due to the detection of PCR artefacts such as truncated elongations, primer concatenamers and other problems related to the specificity of the PCR reaction, as well as to the known imprecision of the Taq I Polymerase enzyme.

Description of the Invention

The nucleic acids in the analyte preferably comprise double-stranded DNA. They may be amplified by the action of DNA polymerase which is capable of synthesising in the 5'-3' direction a complementary strand from a template, in the presence of a primer which is complementary to an extreme portion of the single-stranded analyte sequence. Preferably, amplification occurs for both strands of the analyte sequence, and the DNA polymerase is heat-stable.

The amplified strands may then be denatured. Advantageously, the denaturation occurs by means of incubation, e.g. at a temperature between 90 and 97°C, or in the presence of NaOH.

Preferably, the capture probe is conjugated to a hapten such as fluorescein isothiocyanate (FITC). Then, separation is by means of anti-hapten antibodies, e.g. anti-FITC, which are immobilised on a solid phase, preferably magnetisable microparticles which are attracted onto magnetic plates. The liquid phase containing free detection probes may be removed by washing.

The detection probe is preferably conjugated to an enzyme or biotin. Detection is then conducted by means of incubation with a substrate which is specific for the enzyme, preferably chromogenic, termination of the reaction, e.g. by adding a stop solution, and colorimetric reading of the solution itself. Preferably, the enzyme is an alkaline phosphatase, the specific chromogenic substrate is phenolphthalein monophosphate, and the colorimetric

reading is carried out at a wavelength of 554 nm. A probe that is conjugated to biotin may be detected by means of avidin conjugated to an enzyme.

In general, the present invention is particularly useful for the detection of nucleic acid sequences comprising contiguous DNA segments from different chromosomes, or from different zones of the same chromosome. This may be the result of any of the following biological processes: chromosomal translocation, transposition, intergene or intragene recombination, insertion, deletion or point mutation.

The invention is particularly adapted to the detection of such biological processes that are correlated to pathological states of the organism which the analyte sequence comes from. Thus, the translocation may be correlated to a neoplastic state, as for instance those related to T and B lymphocytes; for example, the translocation may be t(14;18), the analyte sequence bcl-2/J_H, and the neoplastic state follicular lymphoma. In this case, the sequence of the analyte DNA contains the recombination point of two human chromosomes 14 and 18, and the probes bind to either side of the target sequence on the same DNA strand, e.g. the negative strand. It is important that the primers be of such length and composition as not to allow hybridisation to occur with themselves or with portions of the analyte DNA segment which is complementary to the other primer. Accordingly, the extension products are synthesised employing a DNA polymerase, which is preferably heat-stable, and extends the terminal portion to the 3' position of each primer.

The extension products are then separated from their templates by means of high temperature denaturation (92-94°C). The passage is repeated through a number of cycles sufficient to increase the amount of the target sequence up to the concentration at which it can be detected. When the amplification cycles are completed, a suitable amount of the analyte sequence is caused to react with a suitable

concentration of NaOH, e.g. 0.08N NaOH, so as to cause denaturation of the double-stranded segment. Alternatively, denaturation can be carried out through exposure of the DNA to a temperature of 94-97°C for 5-10 minutes and then cooling suddenly down to 0°C.

Once denaturation is completed, a second pair of oligonucleotides is employed. These are probes which are different from the primers employed in the amplification procedure and which are both complementary to the same strand of the analyte DNA, in zones which are to those employed for amplification. The probes are added to the reaction mixture at an excess concentration with respect to the analyte sequence. The pair of probes consists of a capture oligonucleotide and of a reporter oligonucleotide. Each probe is conjugated through its 5' end with a reactive group, to provide an appropriate label.

Reper molecules include haptens, enzymes and radioactive labels, or include any substrate that provides a chromogenic, fluorescent or chemiluminescent signal. By way of example, e.g. the report probe is labelled with alkaline phosphatase and the capture probe with a hapten such as FITC.

The capture probe is suitably separated by linkage to a solid phase such as plastics beads, microplates, coated tubes, latex or, preferably, magnetisable microparticles. By way of example, a hapten can be linked specifically by an antibody immobilised on a solid phase, e.g. anti-FITC on magnetisable microparticles.

Next, a neutralising solution, e.g. 0.5 M Tris, pH 7.5, is added to the reaction mixture, in such an amount as to buffer the NaOH and allow the hybridisation of the probes to the analyte DNA to occur.

After a suitable incubation period at a constant temperature, e.g. 30 minutes at +37°C, an excess amount of a solid phase consisting of magnetisable microparticles coated with an anti-FITC antibody which is capable of binding the whole amount of the FITC-labelled separator

probe, both the free and that reacted with the DNA sequence, is added to the reaction mixture, so forming the analyte sequence-probes complex. After a suitable incubation period at a constant temperature, e.g. 10 minutes at +37°C, the reaction tubes are put on a magnetic plate which, in a short time, e.g. 3 minutes, causes the magnetisable particles to settle onto the bottom of the tube itself.

The supernatant is then removed by decantation, by turning the magnetic plate upside down, the magnetised particles adhering to the bottom of the tube. The tubes are then removed from the magnetic plate and the solid phase is resuspended in a suitable washing solution, e.g. 1 ml of 0.075M Tris-buffered saline, pH = 7.5, allowed to settle and decanted again. The washing cycle is repeated as often as is necessary to remove any non-specific binding of the reagents, and in particular of the reporter probe which is conjugated to the enzyme, with the solid phase. During decantation and washing, all those reactants which are not specifically linked to the magnetic particles are removed from the reaction tube.

Next, a suitable amount of a chromogenic substrate which is enzyme-specific, e.g. 200 µl of phenolphthalein monophosphate, is added to the magnetic particles and allowed to react for the time required at a constant temperature, e.g. 1 hour at 37°C. After this period, the reaction is stopped by adding a stop solution, e.g. 750 µl of a Na_2CO_3 solution, pH 12.

The addition of the stop solution causes the formation and the stabilisation of colour, the absorbance value of which is measured at a suitable wavelength, for instance 554 nm, on a colorimeter. A colour development which is significantly higher than that of blank samples indicates that, during amplification, some extension products were formed starting from the specific primers and from the analyte DNA sequence that has acted as a template. In the absence of the analyte sequence, no formation of specific

extension products would have occurred, which products are the only compounds capable of acting as bridges between the magnetic particles and the reporter probe that bears the enzyme capable of generating the signal. A standard curve may be generated, employing known concentrations of the analyte DNA, to give a concentration value for each sample analysed.

The method for conjugating a reactive group to the oligonucleotide probes obviously depends on the group type that is to be employed; generally the preferred bond occurs through the OH group in the 5' position of the oligonucleotide. During automated synthesis of the oligonucleotide, employing phosphoroamidite chemistry, it is possible to introduce an aliphatic amine at the 5' end employing the Aminolink 2 (ABI) reactant or the Aminomodifier II (Clontech) reactant; this amino group can be reacted successively with a specific hapten, for instance FITC or a biotin-hydroxy-succinimide ester, or any other group containing an ester which is activated and capable of reacting with a primary amine.

For conjugation with the enzyme, it is generally preferred to use heterobifunctional reactants such as succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) and 2-iminothiolane (2-IT), available from Pierce. For instance, SMCC is capable of reacting with the primary amine in the 5' position of the reporter probe give a derivative having a maleimido group free; the 2-IT is capable of reacting with the NH_2 groups of lysines of the alkaline phosphatase so as to give a derivative having a free -SH group. The maleimido groups and the -SH group, if caused to react under suitable conditions, react spontaneously so as to form a very stable carbon-sulphur covalent bond. In this way, it is possible to obtain conjugates in which the reporter probe is linked through its 5' end to the alkaline phosphatase through a long and flexible carbon atom chain, keeping the oligonucleotide capability of specifically hybridising with a complementary

sequence unaltered, and keeping also unaltered the capability of the enzyme to interact with its specific substrate, to generate a coloured solution.

Magnetisable particles coated with anti-FITC antibodies are commercially available (from Ares-Serono, Advanced Magnetics) or they can be prepared by well known procedures. Specific substrates for the phosphatase and stop solutions are also commercially available (from Sigma).

The extension products can be generated by the exposure of the primers, hybridised to their templates, to a DNA polymerase which is preferably heat-stable, e.g. the Taq polymerase disclosed in EP-A-0258017. The DNA polymerase will replicate the sequence of the template, so synthesising some fresh DNA from the primers in the 5'-3' direction. A heat-stable polymerase is preferred, but it is not indispensable because the simplest way of denaturing the double-stranded extension product is by exposure to high temperatures (about 95°C) during the cycles of the PCR, as disclosed in US-A-4683202. By employing different procedures for denaturating the extension products, other polymerases can be used, including the Klenow fragment.

Specifically with reference to detecting the t(14;18) bcl-2 (J_H) translocation, but potentially of more general applicability, it has been found that amplification of either the Major Breakpoint Cluster Region (MBR) or minor cluster region (mcr) can be performed at the same time, depending upon which target is present, using a mixture of primers (3 in total). These primers are respectively specific for (i) the J_H region on chromosome 14; (ii) the MBR region on chromosome 18 (within the 3' untranslated region of the bcl-2 gene); and (iii) the mcr region on chromosome 18 in a region more than 20 Kb downstream from the bcl-2 second exon. Preferred primers of these types, which do not interfere with each other and which yield the same efficiency of amplification for both the MBR and the mcr sequences, are the J_H primer shown as SEQ ID. No. 3,

the MBR primer shown as SEQ ID No. 1, and the mcr primer shown as SEQ ID No. 2 (see Sequence Listing, below).

Following amplification, either the MBR or the mcr-amplified sequence can be detected using specific reporters. Further, it is known that six J_H regions are present in the IgH locus. To be able to detect each individual J_H region that may be randomly involved in the t(14;18) chromosomal translocation, a mixture of six modified oligonucleotides is preferably used. Each oligonucleotide is complementary to one of the six specific J_H regions; they have the respective sequences shown as SEQ ID Nos. 4-9.

Each of these oligonucleotides is modified at both the 3' and 5' end with a NH_2 group during the automated synthesis. Each reporter is thus conjugated at both the NH_2 groups with FITC, and HPLC-purified. The use of 3' and 5' conjugation increases the system sensitivity.

The FITC-conjugated oligonucleotide acts as a capture probe, because it reacts with the anti-FITC coated magnetic particles during the detection assay. The mixture of the six conjugated oligonucleotides is used in the detection of both MBR and mcr-amplified sequences. The determination of which breakpoint is present is made possible by the use of specific probes for either the MBR or the mcr region of the bcl-2 gene.

Both the MBR and mcr reporter oligonucleotides are modified at both the 3' and 5' end with a NH_2 group during the automated synthesis. They are then conjugated to the enzyme alkaline phosphatase and purified as described above. The enzyme-conjugated oligonucleotides act as "signal generating" probes.

The modified oligonucleotides used as reporters (after being conjugated to alkaline phosphatase) are shown as SEQ ID Nos. 10 and 11 (MBR and mcr reporter probes, respectively). The NH_2 modification at both ends of the oligonucleotides increases the amount of enzyme that can be

linked to the probe and subsequently the sensitivity of the detection method.

It is preferred that the reaction buffers in which either the MBR or the mcr detection probes are dissolved differ slightly from each other, in order to account for the different lengths of the amplified sequences (200 bp for the MBR and 400 bp for the mcr). Also the initial dilution of the PCR samples may vary for the two targets (for example, actual dilution for the MBR is 1:10; for the mcr 1:4), as does the incubation time for the hybridisation step (for example, 30 minutes at +37°C for MBR and 15 minutes at +37°C for mcr).

The following description constitutes specific embodiments of the present invention. The "Reagents" illustrate a kit of the invention and the "Recommended Procedures" illustrate the process of the invention. These are taken from the instructions associated with a kit marketed under the trade name C-TRAK FL by Raggio-Italgene S.p.A. This kit is specifically designed for in vitro research use, for the detection of the t(14;18) (q32;q21) chromosomal translocation in frozen biopsies, paraffin-embedded tissues, peripheral blood and bone marrow. Prior DNA isolation can be conducted by standard methods, as described by Maniatis et al in "Molecular Cloning, A Laboratory Manual", 2nd ed. pub. Cold Spring Harbour.

Reagents

Each kit contains sufficient PCR primers to run 25 amplifications. These amplifications can be subdivided into a maximum of 5 runs - 3 samples plus 2 PCR controls in each run.

There are sufficient detection reagents to assay all the amplified samples for both the MBR and mcr, as well as for running the necessary detection controls.

The following reagents are provided:

No.1 PCR Primers (J_H; MBR; mcr) 1 vial (lyophilized)
Contains: 3 nanomoles of each primer

To be reconstituted with 300 µl of distilled water (reagent No. 14). Store at -20 °C after reconstitution.

No.2 Sample Diluent 1 vial (15ml)
Contains: Tris/EDTA (TE) buffer pH 7.5

Ready to use.

No.3 Denaturing Solution 1 vial (3.5ml)
Contains: Diluted NaOH/SDS/EDTA

Ready to use. Store at room temperature - DO NOT REFRIGERATE.

No.4 *bcl*-2-MBR Detection probes 1 vial (15.4ml)
Contains: A set of J_H-FITC reporters (J_{H1-6} probes conjugated to FITC) and a *bcl*-2- MBR probe (conjugated to the enzyme alkaline phosphatase) in reaction buffer.

Ready to use.

No.5 *bcl*-2-mcr Detection probes 1 vial (15.4ml)
Contains: A set of J_H-FITC reporters (J_{H1-6} probes conjugated to FITC) and a *bcl*-2 mcr probe (conjugated with the enzyme alkaline phosphatase) in reaction buffer.

Ready to use.

No.6 Separation Reagent 1 vial (15.4ml)
Contains: A suspension of anti-FITC coated paramagnetic beads in Tris buffered saline.

Ready to use BUT FIRST RE-SUSPEND, IMMEDIATELY PRIOR TO USE.

No.7 Wash Solution (20x concentrate) 1 vial (13.2ml)
Contains: Tris buffered saline

To be made up with 250ml of distilled water.

No.8 Substrate Solution 2 vials (15.4ml each)
Contains: Phenolphthalein monophosphate in triethanolamine buffer.

Ready to use. DO NOT EXPOSE TO DIRECT SUNLIGHT.

No.9 Stop Solution 1 bottle (115ml)
Contains: A sodium carbonate/hydroxide solution pH> 12.

Ready to use. CAUTION: GAUSTIC MATERIAL

No.10 t(14;18) Translocation Positive PCR Control 1 vial (60µl)
Contains: DNA extracted from two cell lines carrying respectively the MBR and the mcr t(14;18) translocation, in TE buffer.

Ready to use.

No.11 Negative PCR Control 1 vial (60µl)
Contains: DNA extracted from a cell line NOT bearing the t(14;18) translocation, in TE buffer.

Ready to use.

No.12 t(14;18) Translocation Positive Detection Control 1 vial (480µl)
Contains: Both MBR and mcr amplified sequences, in TE buffer.

Ready to use

No.13 Negative Detection Control 1 vial (480µl)
Contains: DNA extracted from a cell line NOT bearing the t(14;18) translocation translocation, but subjected to "*bcl*-2" PCR amplification, in TE buffer.
Ready to use.

No.14 Distilled Water 1 vial (3ml)
Contains: HPLC grade, distilled water.

Ready to use.

Further Reagents

A. Perkin-Elmer Cetus AmpliTaq DNA Polymerase

B. Perkin-Elmer Cetus 10x Amplification Buffer

C. Perkin-Elmer Cetus $MgCl_2$ Solution

D. Deoxynucleotide Triphosphates
Contains: 25mM solutions of dATP, dCTP, dGTP and dTTP
To be diluted 1:20 with distilled water (reagent E)

Amplification of the *bcl*-2 translocated DNA sequence by the Polymerase Chain Reaction (PCR).

1. Reconstitute the PCR primers (reagent No. 1) with 300µl of distilled water (reagent No. 14), mix vortex for several minutes and spin in a microfuge. Store at -20 °C after reconstitution.
2. Dilute dNTPs (reagent D) 1:20 with distilled water (reagent E) by transferring the contents of the tube (110µl) into a vial in which 2.09ml of distilled water have been pipetted. Aliquot and store at -20 °C.
3. Into an autoclaved tube suitable for a PCR thermal cycler, pipette:
-46.5µl of distilled water (reagent No. 14)
-10.0µl of PCR primers (reagent No. 1)
-10.0µl of 10x Amplification buffer (reagent B)
-7.0µl of $MgCl_2$ solution (reagent C)
-16.0µl of dNTPs solution (reagent D)
-0.5µl of Amplitaq (reagent A) - equivalent to 2.5 units of enzyme
-10.0µl of sample; 1 µg of total DNA
- - - - - - -
100.0 µl final volume

Layer on the top of the solution 50µl of mineral oil, spin in a microfuge and start the thermal cycles.

We suggest the following procedure:

i) prepare a 'master mix' (at 0-4 °C in a 'protected' environment) of all the reagents necessary for the PCR reaction except the DNA sample. Sufficient 'master mix' should be prepared for each of the samples plus the positive and negative PCR controls (plus 5% excess).
ii) pipette the 'master mix' into the PCR tubes at 4 °C.
iii) pipette the DNA samples into their respective PCR tubes.
iv) add the mineral oil.
v) spin in a microfuge.
vi) quickly start the thermal cycles.

The recommended protocol for the Perkin-Elmer thermocycler 9600 includes the use of:
0.5mm thin-walled vials
100µl reaction volume/ 50µl mineral oil
time constant of 12.5 (100µl)
and the following instrument set-up parameters:

No. of cycles	Denaturation		Annealing		Extension	
	T(°C)	time(s)	T(°C)	time(s)	T (°C)	time(s)
1	95.0	120	56.5	30	72.0	30
6	95.0	30	56.0*	30	72.0	20
30	94.0	30	53.0	30	72.0	60**
1	94.0	30	53.0	30	72.0	300

stop and hold at 4 °C
* set temperature decrease = 0.5 degrees/cycle
** set time increase = 1 second/cycle

For other PCR instruments which make use of different vials and different heating/cooling devices, we suggest the following set-up parameters. (NB Each user should optimize these parameters for the instrument they are using).

No. of cycles	Denaturation		Annealing		Extension	
	T(°C)	time(min)	T(°C)	time(min)	T(°C)	time(min)
1	94.0	5	53.0	2	72.0	3
5	94.0	1	53.0	2	72.0	3
30	92.0	1	53.0	2	72.0	3
1	92.0	1	53.0	2	72.0	10

stop and hold at 4 °C

Detection of the amplified DNA sequence

Before use, bring all the reagents to room temperature, gently but thoroughly mixing them using a rolling or orbital mixer, or equivalent device. (Take them out of the refrigerator at least half an hour before use.)
Do not expose to direct sunlight.
Do not expose to direct heat sources.
NB The detection of the MBR and mcr must be carried out in two separate experiments.

1. Add the entire contents of the Wash Solution - 20x concentrate (reagent No.7) to 250ml of distilled water and mix well.
2. Make sure the PCR sample is clear, if not then vortex mix and spin in a microfuge.
3a. To detect the MBR, dilute each PCR reaction mixture 1:10 with Sample Diluent (reagent No.2), using at least 20µl of sample, vortex mix and spin in a microfuge.
3b. To detect the mcr, dilute each PCR reaction mixture 1:4 with Sample Diluent (reagent No.2), using at least 20µl of sample, vortex mix and spin in a microfuge.
4. Take the tube rack out of the magnetic separator. Place in the rack two reaction tubes for each of the diluted PCR samples and for both the Positive Detection Control (System reagent No. 12) and the Negative Detection Control (reagent No.13). Label the tubes appropriately.
5. Pipette in duplicate 20µl of each sample and control into their respective tubes, making sure that you pipette into the bottom of the tube.
6. Using a multipipette, dispense into each tube 20 µl of Denaturing Solution (reagent No. 3).
   Note: The addition of the Denaturing Solution to all tubes should be completed within 3 minutes.
7. Shake the rack manually, using a side-to-side motion for some seconds, making sure the samples come into contact with the Denaturing Solution.
8. Incubate the rack of tubes in a waterbath at 37 °C for 10 minutes.
9. Using a multipette, dispense 200µl of either MBR Detection Probes (reagent No.4) or mcr Detection Probes (reagent No. 5), into each tube.
10. Shake the rack manually, using a side-to-side motion for some seconds.
11. Incubate the rack of tubes in a waterbath at 37 °C for either 30 minutes when detecting the MBR or for 15 minutes when detecting the mcr.

12. Dispense 0.1ml of thoroughly mixed Separation Reagent (reagent No. 6) into each tube.
Notes:
- **do not** use a magnetic stirrer to mix the Separation Reagent.
- the magnetic antibody suspension must be thoroughly mixed before use to ensure a uniform suspension of magnetic particles. After pipetting into 10 to 20 tubes swirl the vial.
- the addition of the Separation Reagent to all tubes should be completed within 3 minutes.

13. Cover the tubes. Gently vortex mix the rack using a multi-vortex. Alternatively, shake the entire rack using a side-to-side motion.
Note: gentle but complete and simultaneous mixing is critical to assure good assay performance.

14. Incubate the rack of tubes in a waterbath at 37 °C for 10 minutes.

15. Slide the rack of tubes into the magnetic separator and allow magnetic sedimentation to occur for 4 minutes, making sure all the tubes are in contact with the surface of the separator.

16. Decant the supernatant from all the tubes by inverting the separator in one large, slow, circular movement. Place the inverted separator on absorbent paper in a tray and hit the base of the separator firmly several times to dislodge any droplets of liquid adhering to the sides of the tubes.
Notes:
- a loss of magnetic black particles indicates incorrect decanting technique.
- try to avoid excessive splashing in order to minimize "amplicon" aerosol formation.
- clean up the area thoroughly immediately after use with 0.5% bleach.
- discard the absorbent paper in a sealed bag.
- do not touch the rim of the tubes with hands/pipettes.
- **be aware** that in this phase of the procedure large amounts of amplified sequences may be present in the reaction tubes which may give rise to serious contamination problems if adequate precautions are not taken.

17. Place the separator upright and add 0.5ml of already diluted Wash Solution (reagent No.7) to each tube.

18. Remove the rack from the separator. Place in a multi-vortex mixer. Vortex vigorously - thorough mixing is essential to ensure good assay performance.

19. Slide the rack of tubes into the magnetic separator. Check to see that all tubes are in contact with the surface of the separator. Wait for 3 minutes to allow particles to sediment magnetically.

20. Decant the supernatant from all the tubes as in Step 15.

21. Repeat Steps 16 to 19 twice (three washing steps in total).
Note: at the end of the magnetic separation step, complete draining of all the tubes is vital to avoid an increase in background signal.

22. Label two tubes for "blanking" the spectrophotometer and place them in the rack.

23. Remove the rack from the separator and pipette 0.2ml of Substrate Solution (reagent No. 8) into each tube, including the blank tubes.
Note: the addition of the Substrate Solution to all tubes should be completed within 5 minutes.

24. Cover the rack with plastic film. Thoroughly mix all the tubes using a side-to-side motion.(**Note:** discard the plastic film with great care as it will be heavily contaminated with amplicons.)

25. Incubate the rack in a waterbath at 37 °C for 60 minutes.

26. Pipette 0.75ml of Stop Solution (reagent No.9) into each tube, including the blank tubes.
Note: it is critical to add Stop Solution at approximately the same rate and in the same sequence, as when adding the Substrate Solution.

27. Slide the rack into the magnetic separator and allow the particles to sediment magnetically for at least 5 minutes.

28. Blank the spectrophotometer at 550nm using the blank tubes and then measure the absorbances (A) for samples and controls.
Note: Samples for which the absorbance exceeds the upper limit of the spectrophotometer should be read at 492nm. A_{550} is approximately equal to 5 x A_{492}, though the precise relationship should be determined for each instrument.

Interpretation of results

The results of the assay are indicated by the absorbance values. Samples which yield an A_{550} which is significantly higher than the PCR Negative Control should be scored as positive.
ie. $(Ax_{550} - 3\ S.D.) > (Ac_{550} + 3\ S.D.)$
where:
x = Sample
c = Negative PCR control
S.D.= Standard Deviation

Expected CV (co-efficient of variation) value for the Negative PCR Controls is approximately 15%, where $CV = S.D./A_{550}$

As a check on the crucial issue of PCR carry-over contamination and false positive results, the A_{550} of the Negative PCR Control should not be significantly different from the A_{550} of the Negative Detection Control. If the A_{550} of the Negative PCR Control does significantly exceed the A_{550} of the Negative Detection Control [i.e. $(Ac_{550} - 3\ S.D.) > (Ad_{550} + 3\ S.D.)$, where d denotes the Negative Detection Control], then the results of the whole test run should be disregarded and actions implemented to avoid further PCR carry-over.

To confirm that both the PCR amplification and the detection procedure have been performed correctly, both the PCR Positive control and the Detection Positive Control must yield A_{550} values within the range indicated in the lot-specific data sheet provided with each kit.

Sensitivity

In our laboratories we have been able to detect the presence of 1 translocated cell in 50,000 cells. A negative result in the translocation assay could occur simply as a result of very low concentrations of translocated cells in the sample. Additionally, when investigating potentially low level occurrence of the t(14;18) translocation, statistical sampling methods should be employed.

Precision

Intra-assay precision of the detection step was determined by measuring A_{550} replicates of the same PCR amplified samples and resulted in an average CV of 8-10%.

Inter-assay precision of the detection step (determined as above) gave a CV of 10-15%.

SEQUENCE LISTING

SEQ ID No. 1

Sequence Type: Oligonucleotide

Sequence Length: 24 bases

Strandedness: Single

Topology: Linear

TGA CCT TTA GAG AGT TGC TTT ACG

SEQ ID No. 2

Sequence Type: Oligonucleotide

Sequence Length: 21 bases

Strandedness: Single

Topology: Linear

GAT GGC TTT GCT GAG AGG TAT

SEQ ID No. 3

Sequence Type: Oligonucleotide

Sequence Length: 20 bases

Strandedness: Single

Topology: Linear

ACC TGA GGA GAC GGT GAC CA

SEQ ID No. 4

Sequence Type: Oligonucleotide

Sequence Length: 27 bases

Strandedness: Single

Topology: Linear

NH_2-AAT ACT TCC AGC ACT GGG GCC AGG GCA-NH_2

SEQ ID No. 5

Sequence Type: Oligonucleotide

Sequence Length: 27 bases

Strandedness: Single

Topology: Linear

NH_2-GGT ACT TCG ATC TCT GGG GCC GTG GCA-NH_2

SEQ ID No. 6

Sequence Type: Oligonucleotide

Sequence Length: 27 bases

Strandedness: Single

Topology: Linear

NH_2-ATG CTT TTG ATG TCT GGG GCC AAG GGA-NH_2

SEQ ID No. 7

Sequence Type: Oligonucleotide

Sequence Length: 27 bases

Strandedness: Single

Topology: Linear

NH_2-ACT ACT TTG ACT ACT GGG GCC AAG GAA-NH_2

SEQ ID No. 8

Sequence Type: Oligonucleotide

Sequence Length: 27 bases

Strandedness: Single

Topology: Linear

NH_2-ACT GGT TCG ACT CCT GGG GCC AAG GAA-NH_2

SEQ ID No. 9

Sequence Type: Oligonucleotide

Sequence Length: 27 bases

Strandedness: Single

Topology: Linear

NH_2-ACG GTA TGG ACG TCT GGG GGC AAG GGA-NH_2

SEQ ID No. 10

Sequence Type: Oligonucleotide

Sequence Length: 27 bases

Strandedness: Single

Topology: Linear

NH_2-TTT CAA CAC AGA CCC ACC CAG AGC CCT-NH_2

SEQ ID No. 11

Sequence Type: Oligonucleotide

Sequence Length: 25 bases

Strandedness: Single

Topology: Linear

NH_2-CGC TCT TGT TGA CTG GCT GGC TTA G-NH_2

CLAIMS

1. A process for detecting a target sequence including a chromosomal abnormality in an analyte, comprising the steps of:

amplifying the target sequence in the analyte;

reacting under hybridising conditions in a homogeneous phase the amplified target sequence with excess amounts of two independently-labelled oligonucleotide probes which are respectively complementary to different regions of the target sequence, wherein one label renders its probe separable and the other label renders its probe detectable; and

separating and detecting any resultant hybrid that has both labels.

2. A process according to claim 1, wherein the amplification occurs through the action of a DNA polymerase which synthesises a complementary chain in the 5'-3' direction from a single-strand template, in the presence of primers which are complementary to regions of the target sequence, and the oligonucleotide probes are internally nested with respect to those regions.

3. A process according to claim 2, wherein both strands of double-stranded DNA are amplified and the DNA polymerase is heat-stable.

4. A process according to any preceding claim, wherein the separable probe is conjugated to a hapten, and separation is conducted by means of anti-hapten antibodies immobilised on a solid phase.

5. A process according to claim 4, wherein the solid phase comprises magnetisable microparticles.

6. A process according to claim 4 or claim 5, wherein the hapten is fluorescein isothiocyanate and the antibody is an anti-FITC.

7. A process according to any preceding claim, wherein the detectable or reporter probe is conjugated to an enzyme or biotin, and the detection comprises incubation with a

specific substrate for the enzyme or with a streptavidin-enzyme conjugate.

8. A process according to claim 7, wherein the substrate is chromogenic and the detection comprises a colorimetric reading after addition of a solution that stops the reaction.

9. A process according to claim 8, wherein the enzyme is alkaline phosphatase, the chromogenic substrate is phenolphthalein monophosphate, and the colorimetric reading is at a wavelength of 554 nm.

10. A process according to any preceding claim, which additionally comprises denaturing the amplified sequence.

11. A process according to any preceding claim, wherein at least one of the labels is not a radio-label.

12. A process according to any preceding claim, wherein the abnormality is associated with a neoplastic condition of the T or B lymphocytes.

13. A process according to any preceding claim, wherein the abnormality is a chromosomal translocation and the probes are complementary to regions on opposite sides of the translocation.

14. A process according to claim 12 or claim 13, wherein the translocation is t(14;18), the analyte sequence comprises the break point bcl-2/J_H, and the neoplastic condition comprises follicular lymphoma.

15. A process according to any preceding claim, for detecting more than one abnormality, wherein two or more target sequences are amplified and/or hybridised simultaneously.

16. A kit suitable for carrying out a process according to any preceding claim, comprising the labelled probes and, optionally, any other means or components defined therein.

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 92/00929

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/68

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 87, no. 22, November 1990, WASHINGTON US pages 8923 - 8927; D.A.NICKERSON ET AL.: 'Automated DNA diagnostics using an ELISA-based oligonucleotide ligation assay' see abstract; figure 1 see page 8924, right column, line 1 - line 13 ---	1
Y	WO,A,8 910 979 (E.I. DU PONT DE NEMOURS AND COMPANY) 16 November 1989 see page 5, line 15 - page 7, line 12; claims ---	1,12-14
Y	WO,A,8 908 717 (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM) 21 September 1989 see page 3, line 15 - page 7, line 27 see page 9, line 6 - page 10, line 8; claims ---	1,12-14
	-/--	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
24 AUGUST 1992	21. 09. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	LUZZATTO E.R.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	EP,A,0 189 628 (THE WISTAR INSTITUTE) 6 August 1986 see page 5, line 4 - page 6, line 25	1,12-15
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 85, July 1988, WASHINGTON US pages 4869 - 4873; M.CRESCENZI ET AL.: 'Thermostable DNA polymerase chain amplification of t(14;18) chromosome breakpoints and detection of minimal residual disease' cited in the application see the whole document	1,14
A	NUCLEIC ACIDS RESEARCH. vol. 17, no. 5, 1989, ARLINGTON, VIRGINIA US page 2142; B.K.DE ET AL.: 'Multiple primer pairs for the detection of HTLV-I by PCR' see the whole document	15

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9200929 SA 59926

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 24/08/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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		AU-B- 577245	15-09-88
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		JP-A- 61173798	05-08-86